ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

Antioxidant properties and metabolites profiling of polyphenol-rich fraction from a folk mushroom, *Macrocybe lobayensis*, using different extractant

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Article History:

Received on: 05.08.2018
Revised on: 14.11.2018
Accepted on: 16.11.2018

Keywords:

Free radical scavenging, HPLC,
Phenol rich extract,
Unique extraction process,
Wild edible mushroom

ABSTRACT

Antioxidant property of natural substances especially polyphenols is of current concern as it can protect human health from various chronic diseases. In this context, mushrooms have repeatedly been informed as a reservoir of several bioactive substances; although they still represent an almost untapped group. One such less explored fungus is *Macrocybe lobayensis* that has indigenously been accepted as gastronomic delicacy along different states of India. Thus, the present work was designed for the determination of free radical scavenging activity of polyphenol-rich fraction isolated from the taxon and for this purpose, a quite different extraction process has been followed to maximize yield. Initially, the formulation was characterized quantitatively and qualitatively to estimate therapeutic substances. Further, the extract was subjected to seven *in vitro* model systems to visualize the magnitude of antioxidant property. The fraction was found to be an assemblage of various components like phenol (pyrogallol>cinnamic acid> ferulic acid), flavonoid, ascorbic acid, β-carotene and lycopene with high recovery percentage. Besides, the formulation was detected to be extremely powerful for scavenging hydroxyl radical, followed by DPPH, ABTS and superoxide radicals. The fraction was also able to chelate metal ion efficiently while the reducing power was found to be moderate in action. Overall, the present study implies that *M. lobayensis* could be used in food; cosmetics, as well as pharmaceutical industry and in that note, the extracting method described herein, can be followed to prepare a fraction with high yield and strong antioxidant property.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v10i1.1877

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INTRODUCTION

In biological systems, a significant portion of oxygen is incompletely reduced during respiration that eventually generates pro-oxidant substances with one or more unpaired electrons known as Reactive Oxygen Species (ROS) (Khatua S *et al.,* 2013). These highly toxic molecules include oxygen-centred radicals, such as hydroxyl and superoxide along with some non-radical derivatives, like hydrogen peroxide and singlet oxygen. To protect from their adverse effect, human cells generate few enzymes that can carry out stepwise one-electron reduction. However, the reaction is thermodynamically less favourable, and the condition becomes more opposing when external factors like smoking, exercise, diet etc. initiate radical formation (Singh S *et al.,* 2008). As a result, the level of ROS exceeds the defence mechanism under certain circumstances that breaks redox homeostasis. The resulting oxidative stress can affect many cellular functions as these

radicals often attack nearby chemical compounds to search out ways of pairing up their electrons. It can lead to damage of cellular nucleic acids, proteins and lipids resulting in degenerative diseases such as cataracts, cancer, cardiovascular disorders, diabetes mellitus, immune system decline, brain dysfunction, inflammation and renal failure, among others (Sánchez C., 2016). In this background, antioxidants have been the focus of recent research as they significantly stabilize free radicals by retarding the oxidation process. Several synthetic commercial antioxidants namely butylated hydroxyanisole (BHA), tert-butyl hydroquinone (TBHQ) and butylated hydroxytoluene (BHT) have been used in pharmaceutical trades. Although, they are now under strict regulation due to their potential human health hazards (Jayakumar T *et al.,* 2011). Hence, the search for natural antioxidants would be a promising alternative for the therapeutic industry not only for their harmless effect but also due to low cost and compatibility with dietary intake (Vidović SS *et al.,* 2010).

Several such compounds have been identified from countless biological sources and among them, polyphenols have currently gained importance due to their large array of physiological properties. This diverse secondary metabolite group is comprised of simple phenols such as phenolic acid and complex structure like flavonoids (Martins S *et al.,* 2011). They are characterized by an aromatic ring consisting of one or more hydroxyl groups which make them ideal for free radical scavenging. Thus, they can act as free radical scavengers (donating hydrogen to free radicals involved), reducing agents (electron donators), singlet oxygen quenchers or metal ion chelators (Blokhina O *et al.,* 2003).

Edible mushrooms have long been recognised as reservoir of different types of phenolic compounds that are responsible for the prevention of a multitude of disorders. Thus, the usage of them is gradually extending up not only as a dietary constituent but also in the field of pharmaceuticals, nutraceuticals and cosmeceuticals for mankind (Rathore H *et al.,* 2017). Studies suggest that macrofungi possess more than 100 therapeutic functions of which the key medicinal property is the antioxidant effect (Valverde ME *et al.,* 2015). Till date, many studies have been published reporting free radical scavenging potency of mushrooms including *Pleurotus ostreatus* (Patra S *et al.,* 2013, Mitra P *et al.,* 2013), *Pleurotus florida* (Saha S *et al.,* 2013), *Russula albonigra* (Nandi AK *et al.,* 2014), *Macrocybe crassa* (Khatua S *et al.,* 2014, Acharya K *et al.,* 2015b), *Ramaria aurea* (Khatua S *et al.,* 2015b), *Grifola frondosa* (Acharya K *et al.,* 2015a), *Macrolepiota dolichaula* (Samanta S *et al.,* 2015), *Macrocybe gigantea* (Khatua S *et al.,* 2016, Chatterjee S *et al.,* 2016), *Pleurotus djamor* (Acharya K *et al.,* 2017b), *Laetiporus sulphureus* (Acharya K *et al.,* 2016, Khatua S *et al.,* 2017e), *Meripilus giganteus* (Acharya K *et al.,* 2017a), *Russula alatoreticula* (Khatua S *et al.,* 2017b), *Russula senecis* (Khatua S *et al.,* 2017a) etc. In spite of the increasing research, macrofungi still remain a relatively almost unexplored group.

Macrocybe lobayensis is one such less examined fungus that has long been worshipped as food in different states of India. Recent investigation has described the mushroom as a versatile source of bioactive components with multi-dimensional therapeutic properties like immunomodulation and anticancer effect. Besides, free radical scavenging and antioxidant potential of methanol fraction prepared from its basidiocarps have also been established in our previous publication (Khatua S *et al.,* 2017d). However, the present study was focused on the isolation of a polyphenol-rich formulation from *M. lobayensis* to maximize recovery percentage and antioxidant activity. Accordingly, a modified extraction process has been followed herein by using both organic and inorganic solvents at a specific combination to accommodate a range of secondary metabolites with different polarities. The prediction was confirmed by characterizing the fraction qualitatively and quantitatively using spectrophotometry and HPLC techniques.

MATERIALS AND METHODS

Standards and reagents

Ferric chloride, 2-deoxy-D-ribose, hydrogen peroxide, thiobarbituric acid (TBA), trichloroacetic acid (TCA), L-methionine, nitroblue tetrazolium (NBT), riboflavin, ferrous chloride, ferrozine, potassium ferricyanide, sodium persulfate, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$), ammonium molybdate, 2,6-dichlorophenolindophenol (DCPIP), ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu, ascorbic acid and oxalic acid were procured from Himedia, Mumbai, India. Eleven standards such as gallic acid, vanillic acid, ferulic acid, chlorogenic acid, myricetin, *p*-coumaric acid, quercetin, salicylic acid, pyrogallol, cinnamic acid and kaempferol were purchased from Sigma Aldrich (MO, USA).

Mushroom collection and authentication

Fresh fruit bodies of *M. lobayensis* were gathered from the coastal region of West Bengal, India during the rainy season and identified following standard literature (Pegler DN *et al.,* 1998). A representative specimen (CUH AM 483) was

deposited in the same department (Pradhan P *et al.,* 2015). Collected basidiocarps were cleaned and dehydrated by a field drier at 40°C. Desiccated samples were further crushed using an electric blender, sieved using 160 mesh and maintained in an airtight container.

Preparation of polyphenol-rich extract

Powdered basidiocarps of *M. lobayensis* were extracted with ethanol overnight to discard the alcohol-soluble constituents such as coloured products, fat, small organic constituents like terpenoids and steroid. After air drying, the filtrate was extracted by stirring with boiled distilled water for 7 hrs. The residue was filtered approximately 4 volume ethanol was poured to the supernatant slowly to precipitate polysaccharide and kept at 4°C. Further, the precipitate was collected by centrifugation and discarded, while the supernatant was concentrated in a rotary evaporator under reduced pressure (Rotavapor R-3, Butchi, Switzerland) (Khatua S *et al.,* 2015a). The concentrated polyphenol enriched extract of *M. lobayensis* was kept at 4°C until further analysis. The percentage of yield was estimated based on dry weight:

$$\text{Yield (\%)} = (W_1 \times 100)/ W_2$$

W_1 = weight of fraction after solvent evaporation, W_2 = weight of powdered mushroom

Determination of bioactive mycochemicals

Total phenol content was assessed using Folin-Ciocalteu reagent as per the standard protocol where gallic acid was used as a standard. The result was expressed as µg of gallic acid equivalents per mg of extract. Flavonoid content was estimated using quercetin of concentration ranging between 5–20 µg/ml as standard. Ascorbic acid content was quantified by titration against DCPIP dye. β-carotene and lycopene contents were assessed as per our previous publication (Khatua S *et al.,* 2015a).

Detection of phenolic compounds by HPLC

To identify the constituents, dry extract (0.5 mg) was dissolved in HPLC grade methanol (1 ml). The solution was passed through a 0.2 µm filter membrane and 20 µl of that filtrate was loaded on the HPLC instrument (Agilent, USA). Separation was carried out using a standard protocol as mentioned in our previous literature (Khatua S *et al.,* 2015a). The constituents were recognized based on absorption spectra and retention time of standards.

Determination of antioxidant potentiality

Antioxidant effects of the polyphenol-rich extract were investigated following seven different *in vitro* assay systems such as superoxide radical scavenging, hydroxyl radical quenching, ABTS radical inhibition, DPPH radical scavenging, chelating ability of metal ion, reducing power as well as total antioxidant capacity protocols (Halliwell B *et al.,* 1987, Prieto P *et al.,* 1999, Khatua S *et al.,* 2017c).

Statistical analysis

Data are represented in the present study as mean ± standard deviation of three independent experimentations each in triplicate. Calculations were accomplished using statistical package for Microsoft® Office Excel (Microsoft®, USA) and differences were estimated by means of one-way analysis of variance.

RESULTS AND DISCUSSION

Extractive condition and value

There are many techniques to recover bioactive secondary metabolites as the extractive yield as well as therapeutic efficacy do not solely depend on the specimen; but also on the solvent used for extraction. Various components with different chemical characters and polarities may or may not be soluble in a particular solvent. In that note, the most suitable solution can be aqueous mixtures containing ethanol, methanol, acetone or ethyl acetate. Among them, ethanol is known as a better choice for polyphenol isolation and considered safe for human consumption (Do QD *et al.,* 2014). Therefore, a hydro-ethanol solution (1:4) could be a useful extractant solvent to isolate metabolites in sufficient amount as indicated in our previous publications (Khatua S *et al.,* 2015a, Dasgupta A *et al.,* 2014, Mitra P *et al.,* 2017). Accordingly, the same isolation process has been followed herein to prepare a phenol-rich formulation from *M. lobayensis*. The extract was found to be brown in colour, sticky in nature with noticeable recovery percentage (Table 1). Interestingly, the yield was estimated to be about 1.6 times higher than methanol fraction of *M. lobayensis* (Khatua S *et al.,* 2017d). The observation suggested that the modified extraction procedure might be suitable for leaching out of bioactive substances in significant quantity.

Determination of mycochemical composition

To investigate chemical nature of the extract, different parameters such as phenol, flavonoid, carotenoids and ascorbic acid were quantified (Table 1). Briefly, phenols were estimated as the major constituent which was presented more than two times higher than flavonoids. On the contrary, lycopene and β-carotene were detected in the

lower range and existed almost in an equal quantity of ascorbic acid. Thus, it could be registered that the bioactive elements were existed quantitatively in following the order of phenol> flavonoid> β-carotene> ascorbic acid> lycopene. However, the extract of *M. lobayensis* was detected to be more enriched with secondary metabolites than corresponding fractions of *Amanita vaginata* (Paloi S *et al.,* 2013) and *Termitomyces microcarpus* (Mitra P *et al.,* 2016).

Apart from the spectrophotometric assays, HPLC was carried out for identification of specific phenolic compounds in the studied extract. For that purpose, eleven standards were used and chromatogram indicated pyrogallol as the major phenolic constituent in the fraction (Figure 1). Besides, cinnamic acid was also detected in appreciable quantity, while ferulic acid was identified in minor amount (Table 1). Among these components, pyrogallol and cinnamic acid have also been detected in the methanol extract of *M. lobayensis* (Khatua S *et al.,* 2017d). However, they were presented about six times lesser than the examined phenol-rich fraction.

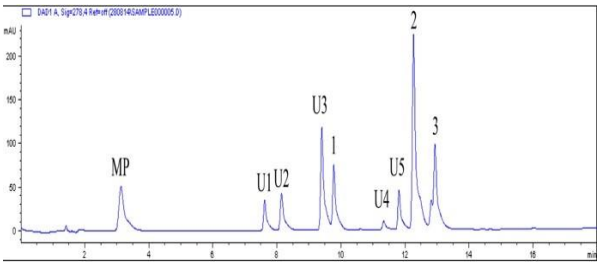

Figure 1: HPLC chromatogram of polyphenol-rich extract isolated from *Macrocybe lobayensis* (MP: mobile phase, peaks 1: ferulic acid 2: cinnamic acid 3: pyrogallol. Peaks designated as U1 to U5 represent unidentified phenols.

Determination of superoxide radical scavenging activity

Generally, superoxide anion radicals ($O_2^{\cdot-}$) are produced within mitochondria of the cell by transfer of one electron to molecular oxygen. However, this relatively weak primary ROS can be transformed to highly damaging components such as peroxyl radical (LOO·), peroxynitrite (ONOO·), hydroxyl radical ($OH^{\cdot}$), singlet oxygen and hydrogen peroxide (Valko M *et al.,* 2007, Huang D *et al.,* 2005). Therefore, it is essential to characterize the scavenging potential of $O_2^{\cdot-}$ by the antioxidants. The method used herein is based on the synthesis of $O_2^{\cdot-}$ by oxidation of riboflavin. As a result, yellow dye, NBT is reduced to produce blue formazan. The decrease in absorbance in the presence of antioxidants designates the consumption of superoxide anion. Eventually, the polyphenol-rich extract from *M. lobayensis* displayed a significant dose-dependent scavenging outline at the examined concentration ranges and results were plotted in Figure 2a. At the level of 100, 300, 500, 700 and 1000 µg/ml the quenching abilities were 11.31%, 28%, 45.32%, 49.43% and 53.47% respectively. However, the synthetic antioxidant, ascorbic acid, presented an excellent function and differences between extract and the control was found to be statistically significant ($p< 0.05$) (Table 2). Based on the outcome, it can be said that phenol-rich formulation of *M. lobayensis* may possess stronger $O_2^{\cdot-}$ radical scavenging potentiality than the corresponding extract of *A. vaginata* (Paloi S *et al.,* 2013).

Estimation of hydroxyl radical scavenging potentiality

Hydroxyl radicals ($OH^{\cdot}$) are considered as the most toxic ROS as they can damage biomolecules immediately after generation (Valko M *et al.,* 2007). Hence, it is necessary to estimate $OH^{\cdot}$ quenching ability of the natural compound and for that purpose Fe^{2+}- ascorbate- EDTA- H_2O_2 model system was adopted. The method is based on Fenton's reaction that generates $OH^{\cdot}$ which in turn generates malondialdehyde (MDA). MDA creates pink chromogen with TBA that can be measured spectrophotometrically. When antioxidant is added to the reactant solution, it disrupts OH formation causing a decrease in colour intensity (Halliwell B *et al.,* 1987). As presented in Figure 2b, the studied extract showed potent $OH^{\cdot}$ scavenging activity which increased gradually with the advent of concentration. The fraction inhibited 18.61%, 43.18%, 59.62%, 68.24% and 78.34% radicals at the level of 10, 30, 50, 70 and 100 µg/ml respectively resulting extremely low EC_{50} value (Table 2). Thus, the extract was detected to exhibit better antioxidant activity than many natural substances like black ear, jin ear and red ear mushrooms (Mau J-L *et al.,* 2001).

Evaluation of ABTS radical scavenging effect

ABTS radical cation ($ABTS^{\cdot-}$) was used in the present study for further evaluation of antioxidant activity of the phenol-rich extract. In this method, $ABTS^{\cdot-}$ were produced by persulfate oxidation of $ABTS^{2-}$ and reduced in the presence of antioxidant substance resulting decolourization (Huang D *et al.,* 2005). Analysis indicated that the fraction possessed strong radical scavenging activity that incremented in a dose-wise manner (Figure 2c). As the concentration ranged from 100, 300, 500, 700 to 1000 µg/ml, inhibition activities of the fraction amplified from 9.69%, 20.21%, 29.69%, 45.17% to 68.24% respectively. However, EC_{50} value of the extract was detected to be higher than ascorbic acid that exhibited about 99% radical quenching activities at those tested concentrations (Table 2).

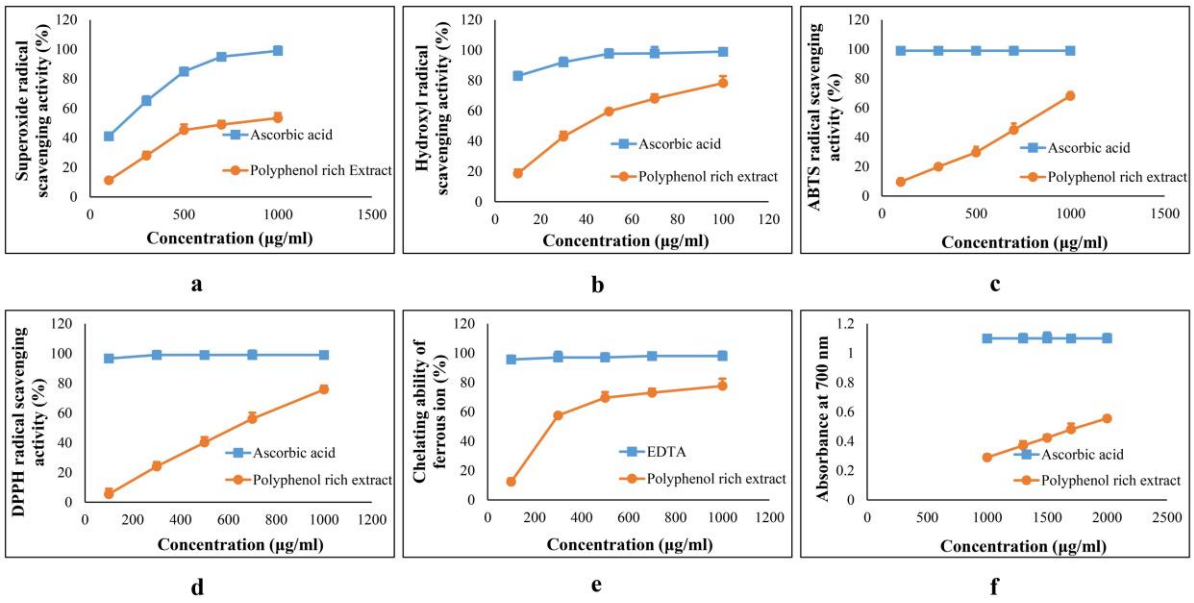

Figure 2: Antioxidant activity of polyphenol-rich extract prepared from *Macrocybe lobayensis*
(a) Superoxide radical scavenging activity (b) Hydroxyl radical scavenging activity (c) DPPH radical scavenging activity (d) ABTS radical scavenging activity (e) Chelating ability of ferrous ion (f) Reducing power

Table 1: Extractive yield and mycochemical composition of polyphenol-rich extract prepared from fruit bodies of *Macrocybe lobayensis*

Parameters	Polyphenol-rich extract
Extractive yield (%)	26.2 ± 7.01
Phenol (µg GAE/mg of extract)	9.28 ± 0.79
Flavonoid (µg QE/mg of extract)	4.15 ± 0.22
β-carotene (µg/mg of extract)	0.7 ± 0.01
Lycopene (µg/mg of extract)	0.54 ± 0.02
Ascorbic acid (µg/mg of extract)	0.58 ± 0.08
Gallic acid (µg/mg of extract)	ND
p-hydroxybenzoic acid (µg/mg of extract)	ND
Chlorogenic acid (µg/mg of extract)	ND
Vanillic acid (µg/mg of extract)	ND
p-coumaric acid (µg/mg of extract)	ND
Ferulic acid (µg/mg of extract)	1.65 ± 0.02
Myricetin (µg/mg of extract)	ND
Salicylic acid (µg/mg of extract)	ND
Quercetin (µg/mg of extract)	ND
Cinnamic acid (µg/mg of extract)	1.38 ± 0.04
Pyrogallol (µg/mg of extract)	51.54 ± 2.45

ND: Not detected

On the contrary, the fraction presented effective antioxidant potential than *Grifola frondosa*, *Lentinula edodes*, *Pleurotus scitrinopileatus*, *Pleurotus eryngii*, *Pleurotus salmoneo-stramineus*, *Trametes versicolor* (Smith H *et al.,* 2015).

Assessment of DPPH radical scavenging activity

The model of scavenging DPPH radical has been a widely used method to evaluate antioxidant activities in a relatively short time compared to other techniques. It is a rapid, consistent and reproducible way to investigate *in vitro* antioxidant effect of pure compounds as well as extracts. The assay is based on reduction of purple coloured DPPH to a stable diamagnetic molecule, diphenyl picrylhydrazine. Antioxidant compounds possess such reducing ability and can donate electron or hydrogen to DPPH. As a result, the reaction solution fades from purple to yellow that can be measured spectrophotometrically. Thus, the degree of discolouration indicates the scavenging potential of antioxidant compounds in term of hydrogen donating ability (Khatua S *et al.,* 2017c). As shown in Table 2, polyphenol-rich extract from *M. lobayensis* exhibited potent radical scavenging activity at the rate of 5.45%, 24.23% and 40.09% at 100, 300 and 500 µg/ml

Table 2: Antioxidant activity of polyphenol-rich extract isolated from fruit bodies of *Macrocybe lobayensis*

Antioxidant parameters		Polyphenol-rich extra t	Standard
EC_{50} value (µg/ml)	Scavenging ability of superoxide radical	827 ± 12.64[a]	123.15±15.63[b]
	Scavenging ability of hydroxyl radical	41 ± 5.67[a]	6.9 ± 0.03[b]
	Scavenging ability of ABTS radical	750.38 ± 12.94[a]	3.18 ± 0.01[b]
	Scavenging ability of DPPH radical	644.85 ± 50[a]	7.69 ± 0.02[b]
	Reducing power	1783 ± 137[a]	18.74 ± 0.01[b]
	Chelating ability of ferrous ion	262.8 ± 11[a]	11.81 ± 0.58[b]
Total antioxidant activity by phosphomolybdenum method (µg ascorbic acid equivalent/mg of dry extract)		10.5 ± 0.79	_

Results are depicted in EC_{50} values (mean ± standard deviation; n = 3) representing 50% of antioxidant activity or 0.5 of absorbance for reducing power assay. Ascorbic acid was used as a standard in superoxide, hydroxyl, ABTS and DPPH radical scavenging methods, reducing power and total antioxidant capacity assays; while EDTA was adopted as a positive control in the chelating ability of ferrous ion technique. Different letters in each row denote significant differences between sample and standard ($p < 0.05$).

concentrations that increased to 56.21% and 75.72% at 700 and 1000 µg/ml concentrations respectively. Whereas standard ascorbic acid showed 99% inhibition at these experimented concentrations (Figure 2d). EC_{50} value of the studied fraction was found to be lower than the phenolic extract of *R. senecis* (Khatua S *et al.,* 2015) and *A. vaginata* (Paloi S *et al.,* 2013).

Determination of chelating ability of ferrous ion

Transition metals like Fe^{2+}, Cu^{+}, Pb^{2+} and Co^{2+} are known to function as catalysts for a radical generation. Chelating agents can stabilize these metals in living systems and thus inhibit the formation of free radicals (Jayakumar T *et al.,* 2011). In this note, chelating ability of the studied fraction was evaluated, and for that purpose, the ferrozine-Fe^{2+} assay system was performed. Ferrozine can react with Fe^{2+} under ethanol or water solution to form a violet complex. While the presence of any chelating agent can disrupt the complex resulting in decrease in colour (Khatua S *et al.,* 2017c). As presented in Table 2, the extract of *M. lobayensis* demonstrated a marked capacity for iron-binding ability which showed a dose-dependent response. At 100, 300 and 500 µg/ml concentrations the fraction presented 12.32%, 57.54% and 69.51% chelating ability that incremented to 73.23% and 77.62% at the level of 700 and 1000 µg/ml respectively. In contrast, EDTA depicted an excellent chelating ability of about 97% at these examined doses (Figure 2e). Remarkably, the methanol extract of *M. lobayensis* exhibited lower potentiality than the studied formulation in term of chelating effect (Khatua S *et al.,* 2017d).

Estimation of reducing power

Further, the ferricyanide/prussian blue protocol was performed to estimate reducing power of the studied formulation. The method is based on the change of yellow colour of reactant solution to green or blue depending on reducing power of the examined sample. Antioxidants can donate electrons that ultimately converts Fe^{+3} in ferric chloride to Fe^{+2}. Subsequent Perl's Prussian blue can be monitored spectrophotometrically and higher absorbance indicates stronger reducing power (Huang D *et al.,* 2005). According to result, extract presented moderate reducing power which augmented with the rise of doses (Figure 2f). At the concentration of 1000, 1300, 1500, 1700 and 2000 µg/ml the fraction showed reducing power of 0.29, 0.37, 0.42, 0.48 and 0.56 respectively indicating better activity than methanol extract of *M. lobayensis* (Khatua S *et al.,* 2017d).

Evaluation of total antioxidant capacity

The phosphomolybdenum method is an efficient way for assessment of the total antioxidant capacity of a component. The protocol is based on reduction of Mo (VI) by the antioxidant compound to Mo (V) which results in the production of green coloured phosphate/Mo (V) complex. Total antioxidant capacity of the phenol-rich formulation was investigated and compared against ascorbic acid. Result exhibited that the antioxidant capacity of 1 mg of extract was equivalent to 10.5 µg ascorbic acid. Phenolic formulation of *T. clypeatus* exhibited a lesser effect than the corresponding fraction of *M. lobayensis* (Mitra P *et al.,* 2017).

CONCLUSION

A polyphenol-rich extract from *M. lobayensis* has been prepared in the present study following an unusual extraction process. The fraction was found to be enriched with different bioactive substances with a high recovery percentage. Besides, the formulation exhibited strong antioxidant potentiality in terms of radical scavenging activity, reduction power and the chelating ability of ferrous ion. Thus, the mushroom could serve as an easily accessible item of food rich in natural antioxidants and the hydro-ethanol extract could probably be used for the manufacture of pharmaceutics.

Acknowledgements

Authors would like to acknowledge the facilities provided by Department of Botany (UGC-CAS Phase VI, VII), University of Calcutta and DST-FIST for instrumental support.

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